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ORIGINAL ARTICLE

Bud vigor, budburst lipid peroxidation, and hormonal changes during bud development in healthy and moribund beech (*Fagus sylvatica* L.) trees

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Abstract

Key message **Despite meristem totipotency, epicormic branches from moribund trees produce buds with less vigor and increased lipid peroxidation compared to those of healthy trees.**

Abstract A better understanding of the factors governing bud vigor and development, and their underlying mechanisms is essential for management of broad-leaved stands, of which oaks and beeches are among the most important species. This study investigated possible differences in leaf bud vigor and development in healthy and moribund beech trees. Leaf buds on shaded branches found in the understory of a natural beech stand in Catalonia (NE Spain) were used for comparison. The vigor, extent of lipid peroxidation, and hormonal profile of leaf buds of healthy and moribund beech trees were investigated. Results showed less bud vigor in moribund trees than in healthy ones. In the former, there was increased lipid peroxidation at budburst. Although endogenous levels of gibberellins, cytokinins, and auxin were significantly influenced by bud development in both tree types, buds of moribund trees showed markedly different dynamics in the endogenous concentrations of auxin, abscisic acid and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. We conclude that moribund trees (a) are less efficient in producing new leaves, as indicated by less bud vigor and greater lipid peroxidation at budburst than healthy trees and (b) show a marked differential hormonal pattern during bud development compared to healthy trees.

Keywords Beech (*Fagus sylvatica* L.) · Budburst · Bud development · Hormones · Moribund trees

Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
DHZ	Dihydrozeatin
DHZR	Dihydrozeatin riboside
GA	Gibberellin
JA	Jasmonic acid
IAA	Indole-3-acetic acid
2iP	2-isopentenyladenine
IPA	Isopentenyladenosine
JA	Jasmonic acid
MDA	Malondialdehyde
RWC	Relative water content
SA	Salicylic acid
Z	Zeatin
ZR	Zeatin riboside

Introduction

Factors governing bud vigor and development, and their underlying mechanisms in broad-leaved stands, of which oaks and beeches are among the most important species, have been the subject of intensive research in recent decades, due to their important implications for forest management (Huttunen et al. 2001). The sprouting of epicormic branches is commonly observed in established beech stands

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(Fink 1980). Epicormic branches are formed from an epicormic bud that has remained latent for a period longer than one year. Although the causes of the formation of epicormic branches or their persistence on the tree are still under investigation, some studies have shown that sprouting is positively related with radial growth (Colin et al. 2012) and also depends on the frequency of existing epicormic branches (Morisset et al. 2012). Additionally, it is often associated with (a) stand thinning, leading to the formation of "light suckers" or (b) increased shading, leading to the formation of "agony branches" or "shade suckers" (Nicolini et al. 2001). Furthermore, epicormic sprouting may be caused by other abrupt changes in a tree's environment, such as severe abiotic constraints (drought, extreme cold), storms, or insect attacks (Meier et al. 2012). In general, any phenomenon that interferes with the basipetal transport of auxin from the apical bud of the main shoot favors epicormic branch formation due to a loss of apical dominance within the tree (Cline 2000).

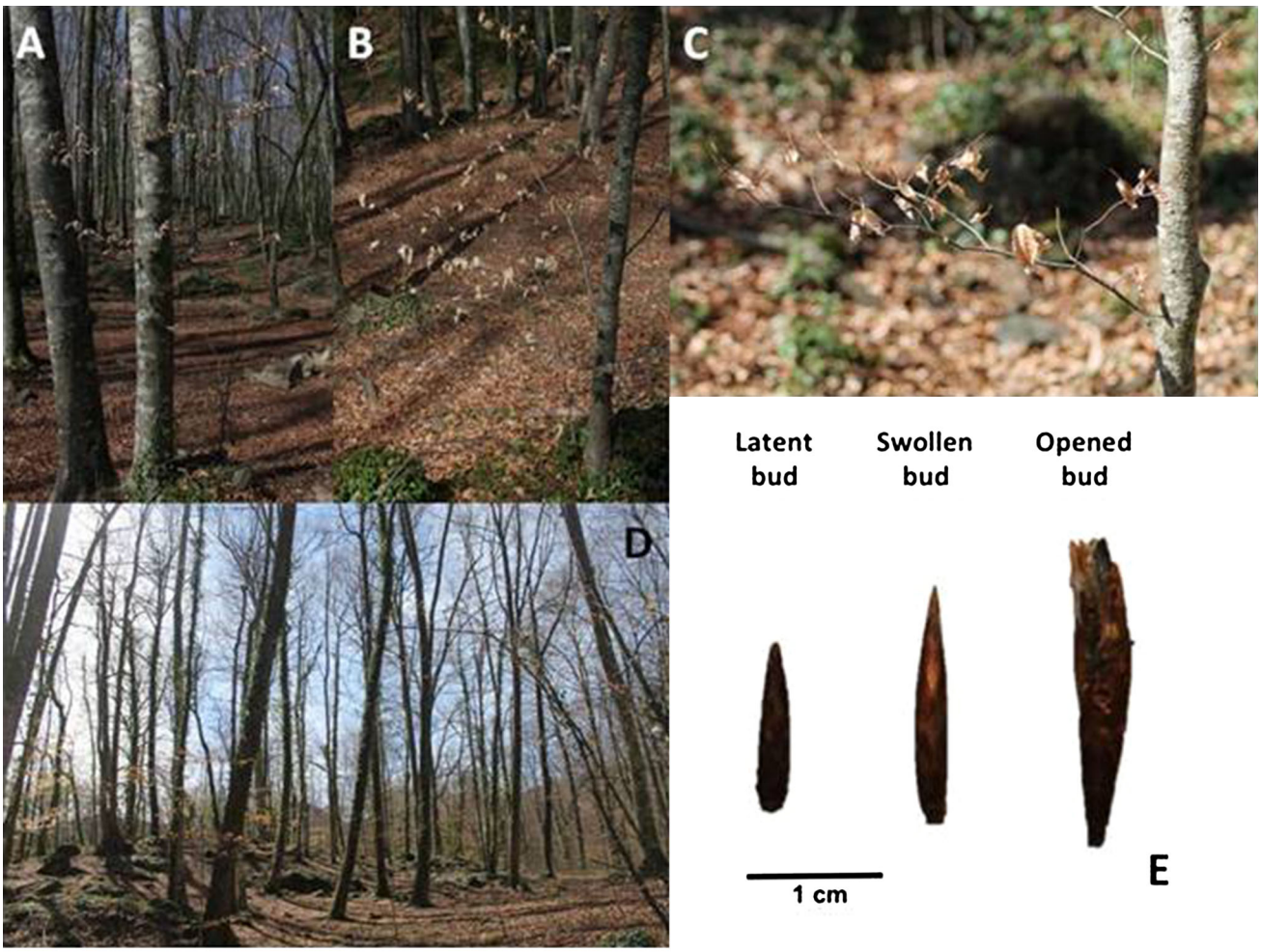
Epicormic branches from moribund trees, according to Kraft's definition (1884, in Lanier 1986), share space and compete for water, nutrients, and light with branches from healthy, young trees, which are being established in the understory of natural beech stands. Although epicormic branch formation has been intensively researched in recent years (reviewed by Meier et al. 2012), the physiology of buds found in epicormic branches of broad-leaved stands has received very little attention. To our knowledge, no studies have compared the physiology of the buds derived from epicormic branches with those from healthy trees found in equivalent climatic conditions in the understory of beech stands. Epicormic branches from moribund trees generally result from damage to the tree, particularly at the apex of the main shoot (Nicolini et al. 2001). Furthermore, due to production of branches instead of trunks, it has been described that epicormic buds are functionally different from apical buds (Del Tredici 2001). Therefore, tissues derived from epicormic branches are expected to show some signs of physiological deterioration. However, theory predicts that a meristem keeps totipotency and the capacity to form new organs almost indefinitely (Verdeil et al. 2007). Given that epicormic branches come from latent buds that were previously undamaged for several years, it is also possible that epicormic branches give rise to tissues with exactly the same physiological status as those formed from healthy trees under the same climatic conditions. Indeed, the formation of epicormic branches serves for the regeneration of the entire tree. Which then of these two will prevail: the meristem (regeneration) effects providing intact tissues or a physiological deterioration linked to the physiology of other parts of the moribund tree? To respond to this question, our research focused on evaluating bud vigor and the extent of lipid peroxidation during budburst, as markers of a possible physiological deterioration, by comparing healthy and moribund individuals in a natural beech stand.

In this study, we hypothesized that bud vigor, lipid peroxidation at budburst, and hormonal patterns during bud development may differ between branches of moribund, old trees and healthy, young ones, the latter showing in general a better physiological status despite the totipotency of meristems. To test this hypothesis, we compared bud vigor for 3 years, measured the extent of lipid peroxidation at budburst and evaluated the variations in the endogenous concentrations of phytohormones during bud development in branches of both tree types in the understory of a natural beech stand.

Materials and methods

Study site, plant material, and sampling

The study site was La Fageda d'en Jordà, a beech stand located near the village of Santa Pau (42°9′N 2 30′E at 565 m. a.s.l., NE Spain). This beech stand is characterized by its growth in volcanic soil. The climatic conditions of the field site are typically continental Mediterranean, with abundant precipitation mostly during autumn and winter and dry, hot summers. Within this beech stand, we distinguished two different types of trees for experiments: (a) healthy, young trees (2–8 cm trunk diameter at breast height -DBH-) and (b) moribund, adult trees (25–45 cm DBH), all of them producing branches in the understory (Fig. 1). Healthy trees presented hierarchical architecture, in which all the structures are grouped in the crown, at the apical part of the tree, while in moribund trees, besides of the crown, epicormic branches are formed all along the main stem, from the stem base to the crown. Healthy and moribund trees coexisted in the same beech stand and were randomly selected from the study area. Buds sampled in healthy trees were from branches that formed part of the apical crown, while in moribund trees they were from epicormic branches. Moribund trees accounted for 10.5 % of the beeches at the sample site. For measurements, twelve healthy and twelve moribund trees were randomly selected and leaf buds were chosen from branches growing 1.5–2 m above the soil surface, so that all buds were always exposed to similar light conditions in the understory. In the case of the moribund trees, these branches have been described as the oldest epicormic branches in the main stem (Nicolini et al. 2001). To obtain buds at different developmental stages, two samplings were conducted at midday. Dormant (latent) buds were sampled on 20 March 2012 ($\approx$400 μmol m^{-2} s^{-1}, 17 °C, 21 % relative humidity), while non-dormant (both closed—swollen—and open) buds were collected on 17 April 2012 ($\approx$500 μmol m^{-2} s^{-1}, 19 °C, 24 % relative humidity). The



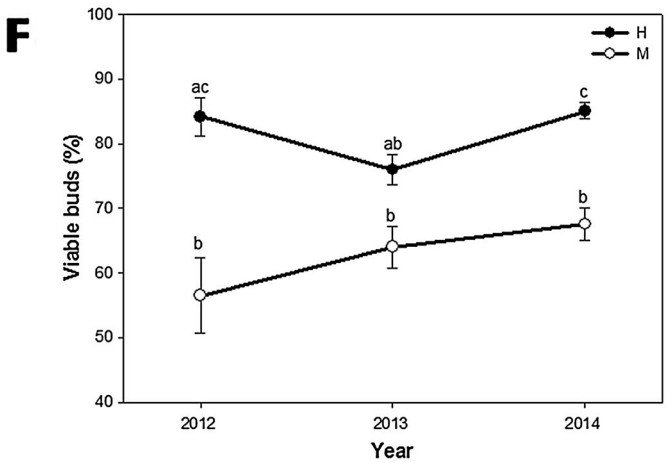


Fig. 1 Photographs illustrating details of the site, trees, and buds used for experiments. **a** Moribund tree; **b** healthy tree; **c** branch from which some buds were sampled; **d** study site, La Fageda d'en Jordà; **e** bud developmental stages sampled in this study. **f** viability of leaf buds in healthy (H) and moribund (M) beech trees during 2012, 2013, and 2014. Data are the mean ± SE of $n = 5$ individuals per tree type, with all buds from three branches per individual tree being analyzed. *Different letters* indicate significant differences ($P < 0.05$)

distinction between the two types of non-dormant buds was performed visually. Closed buds were swollen, but they did not break and no leaves were visible, while opened buds already presented the first leaves (Fig. 1). Bud vigor was measured during 2012, 2013, and 2014, while all other measurements (bud mass and water content, lipid peroxidation,

and hormones) were performed during 2012. For biochemical analysis, samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until analyses.

Bud vigor, biomass, and relative water content

Successful bud development was considered a measurement of bud vigor. Bud vigor (percentage of buds that reached burst) was measured in all buds of three branches of five trees for each tree type. For biomass and water status calculation, buds were transported in a saturated atmosphere to the lab and immediately weighed to obtain the fresh weight (FW). Then, buds were saturated with distilled water for 24 h at 4 °C in darkness to measure their turgid weight (TW). Dry weight (DW) was recorded after oven drying the samples at 80 °C until constant weight was achieved. The relative water content (RWC) was determined as (FW − DW/TW − DW) × 100.

Lipid peroxidation

The extent of lipid peroxidation was calculated by measuring the amounts of malondialdehyde (MDA) equivalents, following Hodges et al. (1999). Bud samples (100 mg) were ground in liquid nitrogen and repeatedly extracted with ice-cold 80 % ethanol (v/v), using ultrasonication for 45 min. The supernatants were pooled, and an aliquot was mixed with the same volume of either −TBA solution containing 20 % trichloroacetic acid and 0.01 % BHT or +TBA solution containing the above plus 0.65 % TBA. Samples were vortexed and heated at 95 °C for 25 min. They were then cooled, and absorbance was read at 440, 532, and 600 nm. MDA equivalents were calculated as $[(A - B)/157{,}000)] \times 10^6$, where $A = [(\text{Abs } 532_{+\text{TBA}}) - (\text{Abs } 600_{+\text{TBA}}) - (\text{Abs } 532_{-\text{TBA}} - \text{Abs } 600_{-\text{TBA}})]$ and $B = [(\text{Abs } 440_{+\text{TBA}} - \text{Abs } 600_{+\text{TBA}}) \times 0.0571]$.

Hormonal profiling

Endogenous concentrations of gibberellins (GAs), cytokinins, auxin, abscisic acid (ABA), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid (SA), and jasmonic acid (JA) were measured by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), following the method described by Müller and Munné-Bosch (2011). Deuterated standards were used to calculate recovery rates for each sample.

Statistical analysis

Statistical analyses were performed with the SPSS package (Chicago, IL, USA). Differences between years or bud developmental stages, and tree types were found by two-way factorial analyses of variance (ANOVA), followed by DMS post hoc tests. When data followed unequal variances (i.e., RWC, MDA, GA_{24}, GA_9, GA_4, GA_{19}, all cytokinins except zeatin, and JA), the Games-Howell post hoc test was applied. Statistical significance was recognized at $P < 0.05$.

Results

Moribund trees show less bud vigor and more lipid peroxidation at budburst than healthy trees

Bud vigor was higher in healthy, young trees than in moribund, adult trees during the three years of study. Maximum differences in bud vigor between tree types were observed during 2012, in which healthy trees had bud vigor above 80 %, while this dropped below 60 % in moribund trees (Fig. 1). Measurements of MDA levels during 2012 revealed increased lipid peroxidation at budburst in both healthy and moribund trees, but especially in the latter (Fig. 2). MDA levels increased from below 2 in dormant buds to 17 nmol (g DW)$^{-1}$ in open buds of healthy trees, while MDA equivalents reached 32 nmol (g DW)$^{-1}$ in open buds of moribund trees, thus indicating increased lipid peroxidation at budburst in the latter. Therefore, fewer buds reached burst in moribund trees (Fig. 1), and the ones that did reach this stage showed increased lipid peroxidation (Fig. 2).

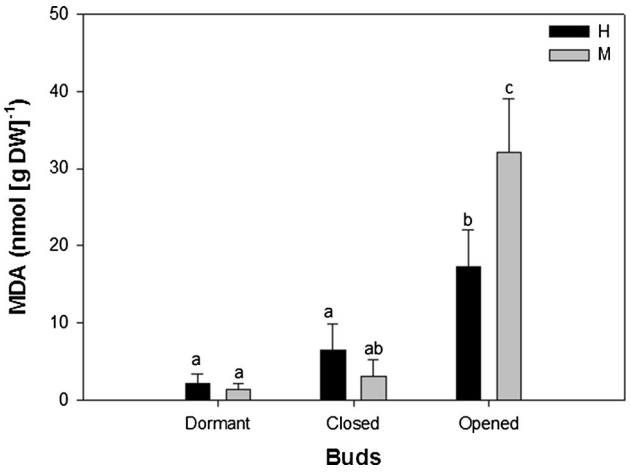


Fig. 2 Levels of malondialdehyde (MDA), an indicator of lipid peroxidation, in dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of $n = 12$ individuals per tree type. Different letters indicate significant differences ($P < 0.05$)

Bud mass and water content analyses did not reveal any difference between healthy and moribund trees (Fig. 3). Bud mass increased in closed, non-dormant buds more than in dormant buds, while it then remained stable until budburst (open and closed non-dormant buds did not differ in mass). The RWC remained at around 80 % throughout bud development, indicating adequate bud water contents in both healthy and moribund trees (Fig. 3).

Hormonal regulation of bud development in moribund and healthy trees

GAs profiling, which included the analysis of GA_4 and GA_1 and their immediate precursors GA_9 and GA_{24} (for GA_4) and GA_{20} and GA_{19} (for GA_1), revealed that GA_4 was the only GA showing significant increases comparing closed, non-dormant buds with dormant buds (Fig. 4). GA_4 concentrations increased around 3–4-fold during bud development in both healthy and moribund trees (Fig. 4). Cytokinin concentrations also increased during bud development, particularly those of zeatin riboside (ZR), whose levels were 5 times greater in closed, non-dormant buds than in dormant buds in both healthy and moribund trees (Fig. 5). The only difference in cytokinins between healthy and moribund trees was observed in the concentrations of dihydrozeatin (DHZ) in dormant buds and dihydrozeatin riboside (DHZR) at budburst, which were 3.7- and 2.2-fold higher, respectively, in healthy trees than in moribund ones (Fig. 5).

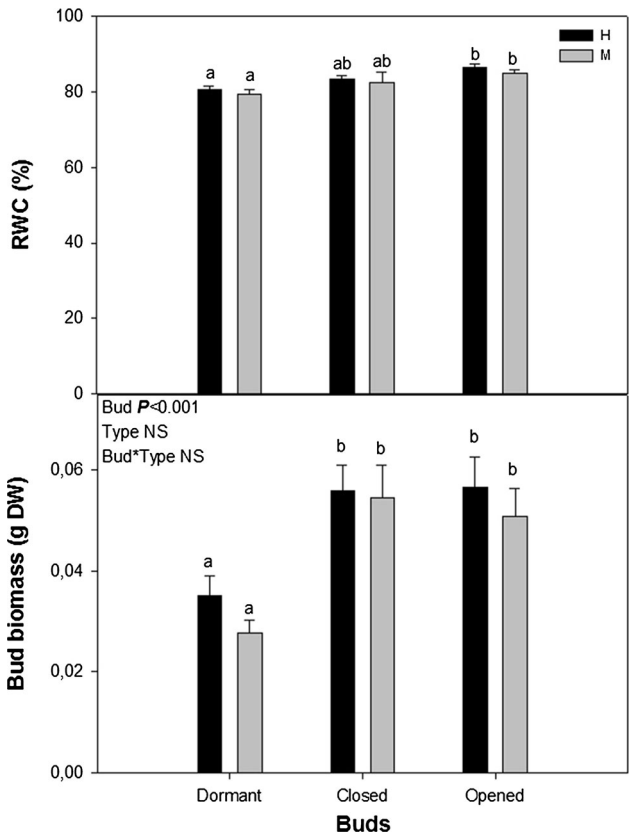


Fig. 3 Bud biomass and relative water content (RWC) of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of $n = 12$ individuals per tree type. *Different letters* indicate significant differences ($P < 0.05$)

In contrast to GAs and cytokinins, major differences in auxin concentrations were observed between healthy and moribund trees. The concentrations of indole-3-acetic acid (IAA) were 3.8-fold higher in dormant buds of healthy trees than in those of moribund trees (Fig. 6). The concentrations of auxin were still higher in healthy trees in closed, non-dormant buds, but not at budburst. Trends were reversed and IAA levels were 40 % higher in open buds of moribund trees than in those of healthy trees (Fig. 6).

No significant differences in ABA concentrations during bud development were observed in healthy trees, but the levels of this hormone decreased significantly after bud break in moribund trees (Fig. 7). Furthermore, ACC levels increased significantly after budburst in moribund trees only (Fig. 7), indicating a difference in the hormonal regulation of bud development between healthy and moribund trees. Although JA levels increased sharply at budburst, no differences were observed between healthy and moribund trees (Fig. 8). SA concentrations did not change with bud development in either healthy or moribund trees (Fig. 8).

Discussion

Hormonal changes during bud development

Hormones play a role in epicormic branch formation and bud development (Meier et al. 2012). Auxins are the hormones considered to be most important in controlling bud dormancy release, since the basipetal transport of auxin in the main shoot prevents lateral shoot development (Cline 2000). Gibberellins are generally recognized as essential regulators of bud break, particularly in buds responding to chilling and photoperiod, such as beeches (Heide 1993; Falusi and Calamassi 2003). As cytokinins play a crucial role in the regulation of cell division and sink strength (Roitsch and Ehneβ 2000), high cytokinin levels will be required in the developing bud for dormancy release and burst. Indeed, the most striking differences in hormonal levels during bud development in our study were observed for GAs, cytokinins, and auxin. The endogenous concentrations of the three hormone classes increased after bud break, which is consistent with a positive role for these compounds that participate in the bud development. Auxins

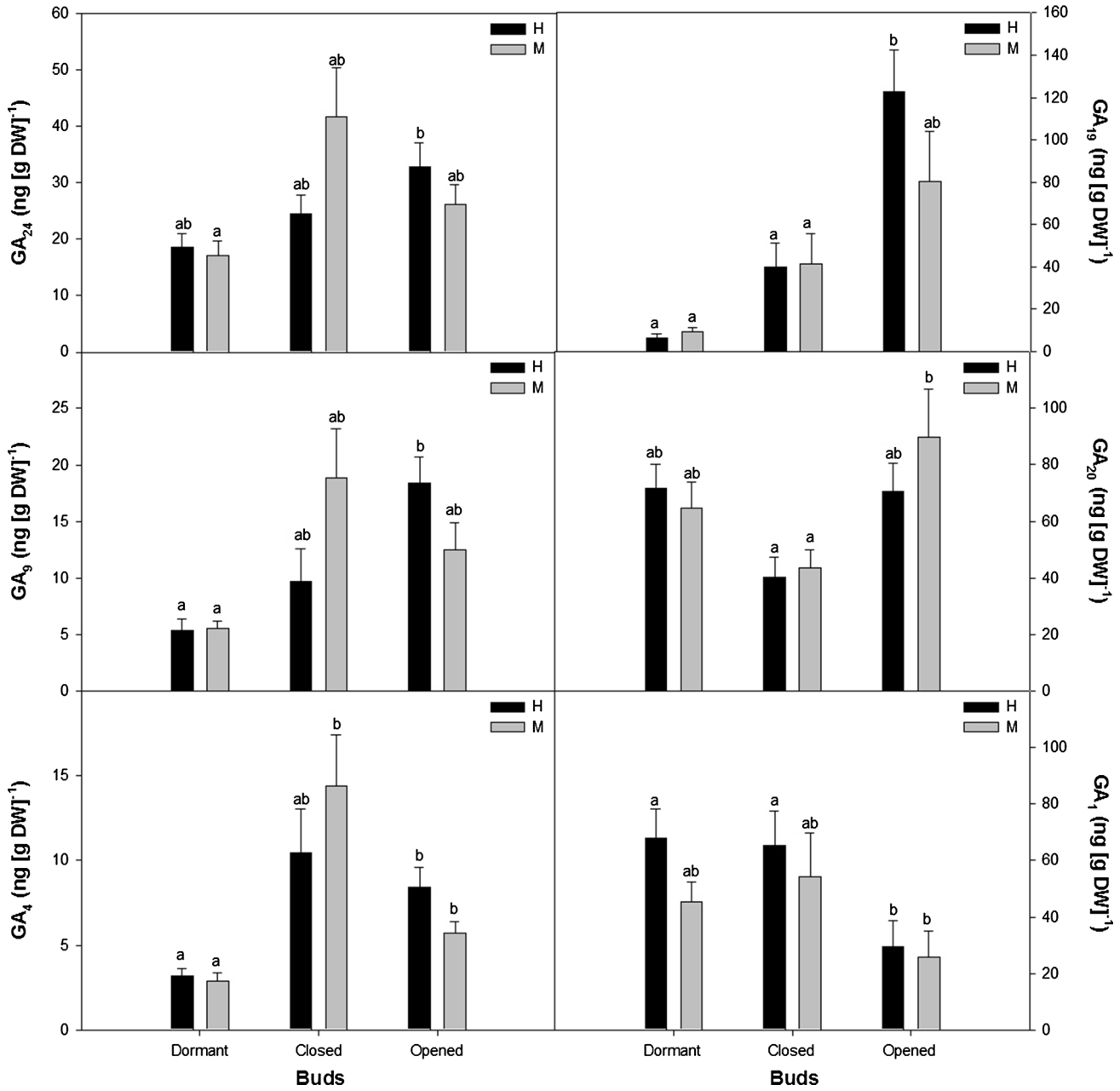


Fig. 4 Endogenous concentrations of gibberellins of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of $n = 12$ individuals per tree type. *Different letters* indicate significant differences ($P < 0.05$)

are considered the most important hormones controlling bud development, since the basipetal transport of auxin in the main shoot prevents lateral shoot development (Cline 2000). Auxin levels were lower in dormant buds of moribund trees than in those of healthy trees, but then auxin concentrations increased sharply at budburst, an indicator of the need to re-establish growth in epicormic branches of moribund trees. Interestingly, cytokinin concentrations increased during bud development, but no differences were observed between moribund and healthy trees. Another important feature is that reductions in ABA concentrations with bud break occurred in buds of moribund trees only. As ABA inhibits growth (Zhou et al. 2003), a reduction in the levels of this hormone, together with GA, cytokinin, and auxin increases, may favor bud development in moribund trees. Thus, the variations in hormone levels during bud development clearly differ between healthy and moribund trees.

Another factor that merits attention is the role of the cytokinin and GA forms that might be active in bud

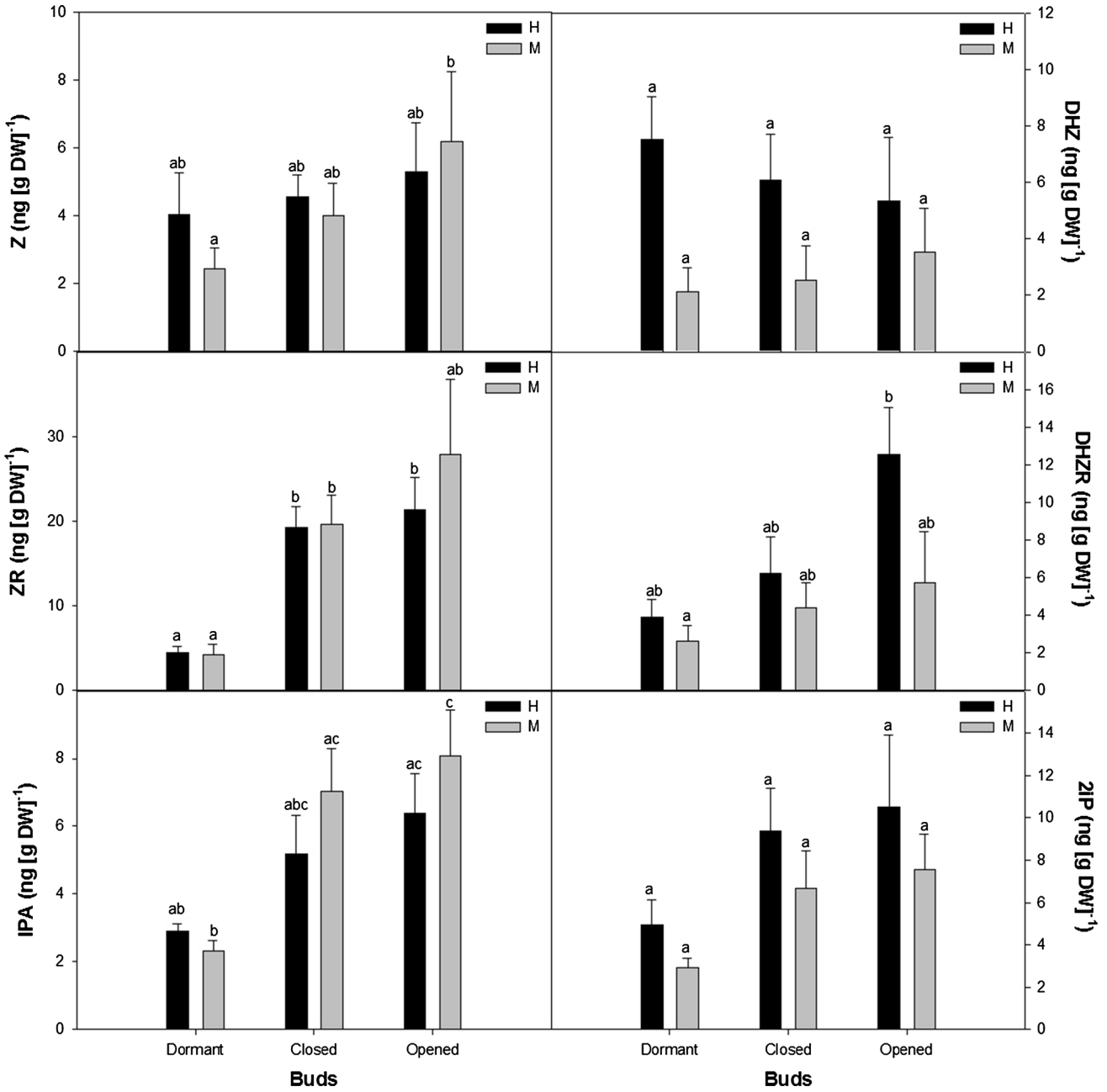


Fig. 5 Endogenous concentrations of cytokinins of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of $n = 12$ individuals per tree type. *Different letters* indicate significant differences ($P < 0.05$). *Z* zeatin, *ZR* zeatin riboside, *IPA* isopentenyladenosine, *DHZ* dihydrozeatin, *DHZR* dihydrozeatin riboside, *2iP* 2-isopentenyladenine

development. Although kinetin applications did not result in increased bud break in beech (Falusi and Calamassi 2003), results reported here suggest that zeatin riboside is the most important cytokinin that participates in bud development. Similarly, GAs profiling revealed that the most active GA that participates in bud development was GA_4. This information is very relevant, since it helps identify the active hormones that participate in the bud development. It can also help in further studies on hormonal applications in beech trees. Also, it is interesting to note the differences in DHZ in dormant buds and DHZR in open buds of healthy and moribund trees, the latter showing lower levels than the former. DHZ and DHZR are thought to be inactive, reduced forms of Z and ZR, respectively, but specific roles for these compounds cannot be ruled out (Van der Krieken et al. 1990; Davies 2010).

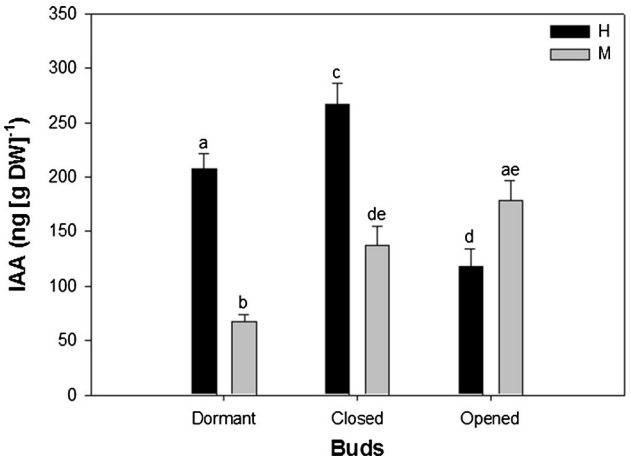


Fig. 6 Endogenous concentrations of the auxin, indole-3-acetic acid (IAA), in dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of $n = 12$ individuals per tree type. *Different letters* indicate significant differences ($P < 0.05$)

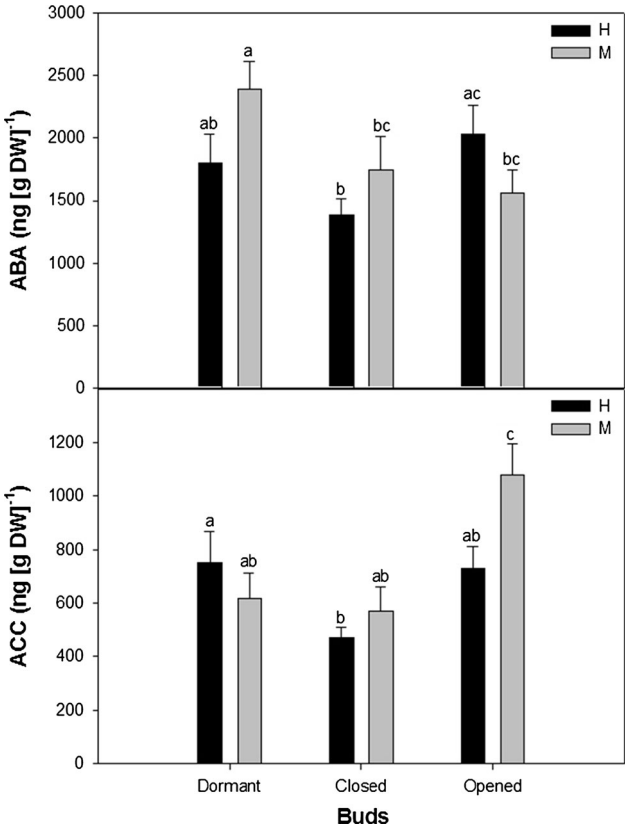


Fig. 7 Endogenous concentrations of abscisic acid (ABA) and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of $n = 12$ individuals per tree type. *Different letters* indicate significant differences ($P < 0.05$)

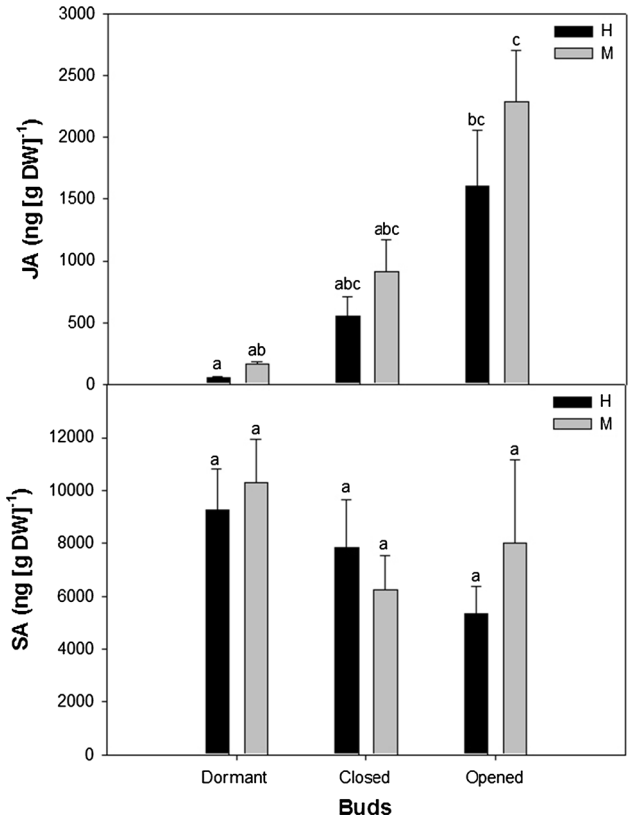


Fig. 8 Endogenous concentrations of jasmonic acid (JA) and salicylic acid (SA) of dormant and non-dormant buds of healthy (H) and moribund (M) beech trees. Non-dormant buds included closed and opened buds (the latter were collected just after budburst). Data correspond to the mean ± SE of $n = 12$ individuals per tree type. *Different letters* indicate significant differences ($P < 0.05$)

Further investigation is consequently required into this differential cytokinin metabolism during bud development and budburst in moribund and healthy trees. Finally, it is worth mentioning that JA and SA are associated with increased resistance to insects and fungi, respectively (Davies 2010; El-Wakeil et al. 2010). Therefore, increases in the levels of JA after budburst could play a role in increasing the bud survival. A more detailed analysis of the hormonal profiling of buds at shorter time intervals will be needed to understand more specifically the role of hormones in bud dormancy release of healthy and moribund trees, which warrants further investigations.

Reduced vigor and increased lipid peroxidation at budburst in moribund trees

Budburst increased the extent of lipid peroxidation. Not only JA concentrations, but also MDA levels increased after budburst in both healthy and moribund trees, thus indicating an increase in both enzymatic and non-enzymatic lipid peroxidation at budburst (Müller et al. 2008). It

should be noted that significant increases in JA and MDA levels were observed in open buds, but not in non-dormant, closed buds, thus indicating that light helps promote lipid peroxidation. Indeed, previous studies have linked photo-oxidative stress with lipid peroxidation in leaves and suggest a link between photoprotection and the trade-off between abiotic and biotic defenses (Demmig-Adams et al. 2013, 2014).

Moribund trees showed increased lipid peroxidation at budburst, indicating an increased physiological deterioration. Not only fewer buds reached burst, but the ones that did had greater lipid peroxidation in moribund than in healthy trees. This is very interesting, since it suggests that epicormic branches of moribund trees are more physiologically deteriorated than newly formed branches of young, healthy trees. Although the branch age may be similar in both tree types, other aspects related to the whole plant, such as a loss of apical dominance, damage to the crown, and/or increased plant size with aging (Mencuccini et al. 2005), may contribute to the observed differences in moribund trees. However, it should be considered that despite reduced vigor, the increased lipid peroxidation and production of lipid-derived signals can be protective and defensive. Both JA and MDA have a role in leaves of protection against stress by modulating both biotic and abiotic stress-related genes (Weber et al. 2004; Müller et al. 2008). In this respect, it should be noted that not only lipid peroxidation-derived signals increased in moribund trees, but also the levels of the ethylene precursor, ACC. Taken together, these results indicate that lipid peroxide-derived signals may act in concert with ethylene to protect epicormic branches of moribund trees. Although results suggest that moribund, old trees are more sensitive to stress than young, healthy ones, our results clearly show that moribund trees activate several defense responses at the biochemical level that undoubtedly help epicormic branches to develop new functional leaves. Therefore, investment of energy in the formation of epicormic branches is very important in moribund trees as it can be considered as a strategy to deal with severe injuries under the prevailing environmental conditions, as well as a strategy for the tree to live longer (Lanner 2002; Ishii et al. 2007). In this respect, it is noteworthy that none of the moribund trees investigated in the present study died during the 3-year study period, so that formation of epicormic branches can be considered a means for survival that help increase the photosynthetic area in damaged trees, thus underlying the great natural regeneration capacity of trees. It is still to be investigated whether or not the frequency of moribund trees increases as plants age. In the present study, moribund trees had a maximum DBH of 45 cm, therefore indicating the study was performed in a young natural beech stand (Di Filippo et al. 2012).

We conclude that (a) gibberellins, cytokinins, and auxin participate in the bud development in beech trees; (b) there is a different hormonal dynamics of bud development in moribund trees; and (c) moribund trees show less bud vigor and greater lipid peroxidation at budburst than healthy trees. Results indicate that moribund trees are less efficient in the production of leaves, but at the same time demonstrate the great regeneration capacity of damaged trees in natural beech stands.

Author contribution statement MJ and SMB conceived and designed the research project: MJ and MM performed the field work; MJ and MM performed the biochemical analyses; MJ analyzed the data; MJ and SMB wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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